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Variability of the inhibition by total immunoglobulin of *in vitro* autoantibody-mediated erythrophagocytosis by mouse macrophages

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Summary

Several autoimmune diseases, mainly autoantibody-mediated, are attenuated by infusion of total IgG (IVIg). The efficacy varies widely from one patient to another. Using an experimental model of *in vitro* phagocytosis of autoantibody-coated erythrocytes by mouse macrophages, we analysed the possible causes for such a variability. Our results indicated that the efficacy of the phagocytosis inhibition depends upon different factors, such as the isotype and the extent of polymerization of the immunoglobulin used for the treatment as well as the genetic background of the mice and the state of macrophage activation that can be influenced by concomitant viral infection. The development of an *in vitro* assay for the phagocytic activity of macrophages might improve the selection of patients susceptible to benefit from IVIg treatment.

Keywords: autoantibodies, autoimmunity, macrophage, phagocytosis, total immunoglobulin

Introduction

Administration of total IgG (IVIg) is used widely to treat patients with certain autoimmune disorders, mediated mainly by autoantibodies [1–8]. Although various modes of action, which could differ from one disease to another, have been proposed [9–24], accumulating evidence points to interaction of the infused IgG with Fc receptors on phagocytic cells as a major mechanism explaining the efficacy of the treatment in immune thrombocytopenia and some cases of haemolytic anaemia [20,25–33]. However, even between patients with a similar pathology the response can vary widely, which can entail a restriction of the use of such an expensive treatment. It would therefore be of considerable interest to understand the causes of these individual variations and therefore to be able to predict for which patients IVIg can be of therapeutic value.

We have previously developed an experimental mouse model that allows for the analysis of IVIg modulation of anaemia induced by passive administration of anti-erythrocyte monoclonal antibodies (mAb) [34–36]. The anaemia was inhibited by total IgG of mouse and human origin and by purified Fc fragments. The protective effect of IgG was shown to be mediated by their interaction with Fc receptors on macrophages. These results correlated well with an *in vitro* analysis of the modulation by total IgG of the phagocytosis of autoantibody-coated erythrocyte by peritoneal macrophages [36]. This *in vitro* assay has now been used to determine the possible causes responsible for the variability in the efficacy of IgG to modulate phagocytosis. Our results indicated that these causes can be found in the IgG preparation itself, such as in its isotype and in its degree of polymerization, as well as in the host, where both genetic factors and the immune environment, in addition to the type of autoantibodies involved, may determine the success of IVIg treatment.

Materials and methods

Mice

Female BALB/c and C3H mice were bred at the Ludwig Institute for Cancer Research by G. Warnier and used at age 6–8 weeks, or were obtained from Iffa Credo (Bruxelles, Bel-

gium). NMRI mice were obtained from the local university animal facility.

Virus

Infection was performed by intraperitoneal injection of approximately 2×10^7 50% infectious doses (ID_{50}) of lactate dehydrogenase-elevating virus (LDV) (Riley strain; from the American Type Culture Collection, Rockville, MD, USA) [37].

Immunoglobulins and antibodies

Human IgG was Gammagard (Baxter, Lessines, Belgium). Monomers, dimers and polymers were purified from Gammagard by chromatography on a Superdex 200 column. No dimers could be detected in the purified monomer fraction. The dimer fraction contained 35% monomers freshly after purification and 52% monomers after freezing. 34–3C anti-mouse erythrocyte mAb was derived from NZB mice [34,35].

IgG1 (Roev and Ho), IgG2 (Kva), IgG3 (Bry) were isolated from sera of patients suffering from multiple myelomatosis by ion exchange chromatography, as described previously [38]. The IgG subclass discrimination was performed by Gm typing [39]. The purity of the isolated, monoclonal IgG preparations was judged to be at least 95% based on Gm typing, agarose gel electrophoresis and gel filtration. Ex160 IgG3 (gift of Dr C. Cambiaso) is an IgG3 human monoclonal antibody of myeloma origin similarly purified by chromatography. Human monoclonal IgG2 and IgG4, here called IgG2-Cal and IgG4-Cal, were obtained from Calbiochem (San Diego, CA, USA). Another human IgG4, here called IgG4-BmD, was obtained from Biomedical Diagnostics (Brugge, Belgium).

In vitro erythrophagocytosis

Erythrophagocytosis was determined *in vitro* as described previously [36]. Briefly, sensitized red blood cells were prepared by incubating 500 µl packed normal erythrocytes with 50 µg mAb in 10 ml phosphate-buffered saline (PBS) containing 2% bovine serum albumin for 30 min at 37°C, then for 1 h at room temperature. Peritoneal cells were collected and allowed to adhere on a tissue culture Petri dish. After washing, they were incubated for 3–16 h with 20 µl washed sensitized red cells in 2 ml Dulbecco's minimum essential medium containing 10% decomplemented fetal calf serum and supplemented with L-asparagine ($0{\cdot}24 \times 10^{-3}$ M), L-arginine ($0{\cdot}55 \times 10^{-3}$ M), L-glutamine ($1{\cdot}5 \times 10^{-3}$ M) and 2-mercaptoethanol (5×10^{-5} M). As indicated, inhibitory proteins were added during this incubation. Cells were washed with PBS and stained with 0·1% o-toluidine in PBS with 10% fetal calf serum. Phagocytosis was expressed as percentage of cells having internalized at least five erythrocytes.

Results

In vitro erythrophagocytosis by peritoneal macrophages

In order to analyse the efficacy of total IgG preparations to inhibit erythrophagocytosis, we used an *in vitro* assay in which peritoneal macrophages were incubated with mouse red cells opsonized with 34–3C, a monoclonal anti-erythrocyte antibody [35,36]. As shown in Fig. 1, erythrophagocytosis of opsonized cells was more efficient than that of uncoated erythrocytes, and LDV infection enhanced the ability of peritoneal macrophages as effector cells, as reported previously [40]. Because separate measurements in the same experimental conditions gave very reproducible data (Fig. 1), subsequent results in experiments with multiple conditions are shown as single measurements obtained with pooled cells from several mice.

Variability related to IgG preparations

Because the interaction of Fc fragment with receptors on phagocytic cells explains the mode of action of total IgG in our model [36], it could be supposed that their immunoglobulin isotype would largely determine their ability to effectively modulate erythrophagocytosis. Thus, the phagocytic activity of murine peritoneal macrophages, derived from BALB/C mice, against target cells composed of 34–3C mAb-coated erythrocytes was measured in the presence of total IgG and of various human mAb of each isotype. For each isotype, two unrelated monoclonal Ig were used. Because the phagocytic activity varies from one experiment to

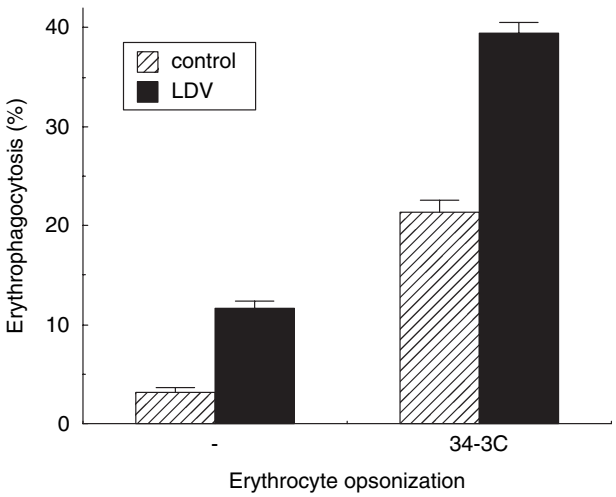

Fig. 1. *In vitro* erythrophagocytosis by peritoneal macrophages from control and infected mice. Peritoneal macrophages from groups of seven BALB/C mice were harvested 3 days after injection of saline (controls) or lactate dehydrogenase-elevating virus (LDV). After pooling, their ability to phagocytose either normal red cells or erythrocytes preincubated with the 34–3C antibody was assessed in five separate measurements. Results are shown as means ± s.e.m.

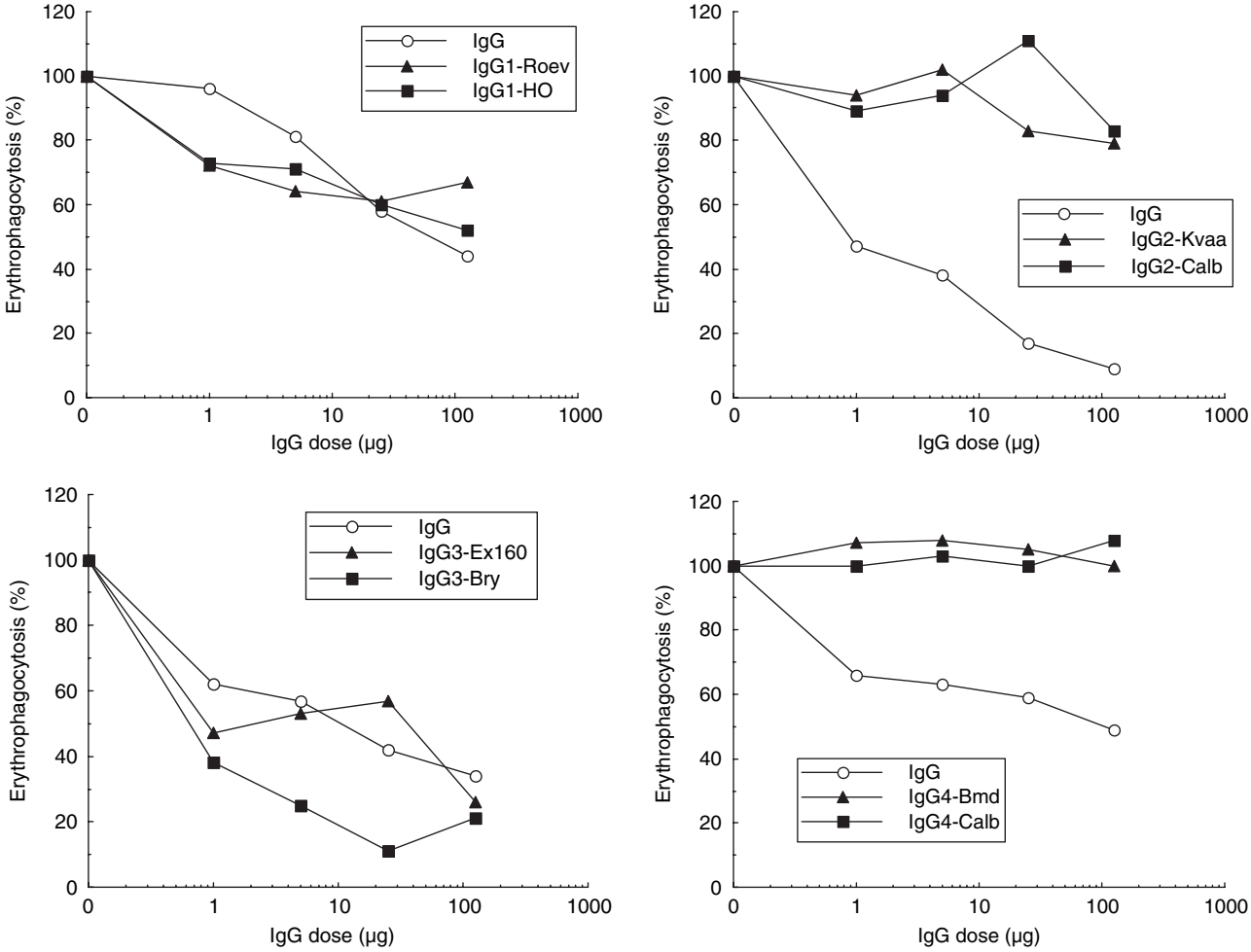

Fig. 2. Inhibition of *in vitro* erythrophagocytosis by human IgG subclasses. Peritoneal macrophages from BALB/c mice were incubated with erythrocytes sensitized with 34–3C monoclonal antibody (mAb) in the presence of various doses of total IgG or of mAb of different subclasses, as indicated. Results of independent experiments are shown as a percentage of erythrophagocytosis observed in the absence of inhibitory IgG.

another, the results of each experiment are presented as the percentage of the maximal phagocytic activity observed in the absence of inhibitory antibodies. As an internal control, phagocytosis inhibition by the same total IgG preparation, that could also slightly differ between independent experiments, was included. Our results, shown in Fig. 2 for representative experiments, indicated that IgG1 and IgG3 mAb suppressed the phagocytic activity of macrophages as efficiently as total IgG. This was observed in six independent experiments with IgG3 and three experiments with IgG1. The best inhibitions were obtained with IgG3 mAbs, with slight variations between different antibodies. In contrast, no inhibition of autoantibody-coated erythrocyte phagocytosis by IgG2 (observed in four independent experiments) or by IgG4 antibodies (in three independent experiments) was observed. Notably, human IgG1 and IgG3 have been shown previously to also decrease anaemia triggered *in vivo* by injection of the same anti-erythrocyte monoclonal autoantibody [36]. Moreover, a mixture of monoclonal antibodies of the different isotypes, in the same proportion as in total IgG (IgG1: 74%; IgG2: 21%; IgG3: 4%; IgG4: 0·4%), induced an *in vitro* inhibition of erythrophagocytosis that was very similar to that observed with these total IgG (data not shown).

We also tested the effect of IgG monomers, dimers and larger polymers that are present in commercial preparations of total immunoglobulins. As shown in Fig. 3 for a typical experiment out of four, the most efficient inhibition of erythrophagocytosis was obtained with dimers. In contrast, larger polymers were far less efficient in this inhibitory activity, although the result of these larger polymers could vary from one experiment to another. Whereas the proportion of IgG1 and IgG2 was similar in monomer and dimer preparations, two to three times more IgG3 were found in dimers when compared with monomers (data not shown).

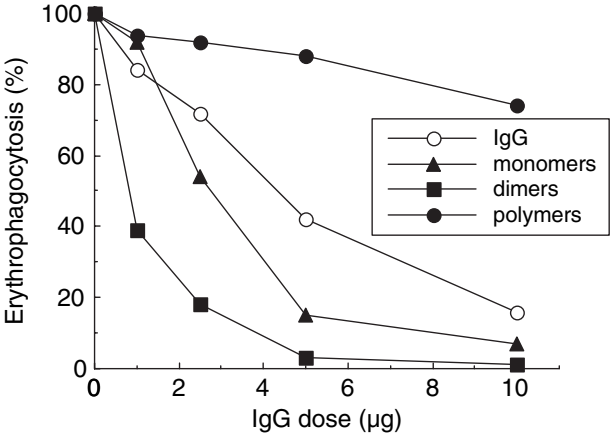

Fig. 3. Inhibition of *in vitro* erythrophagocytosis by human IgG monomers, dimers and polymers. Peritoneal macrophages from BALB/c mice were incubated with erythrocytes sensitized with 34–3C monoclonal antibody (mAb), in the presence of various doses of total IgG or of purified monomers, dimers and larger polymers, as indicated. Results are shown as a percentage of erythrophagocytosis observed in the absence of inhibitory IgG.

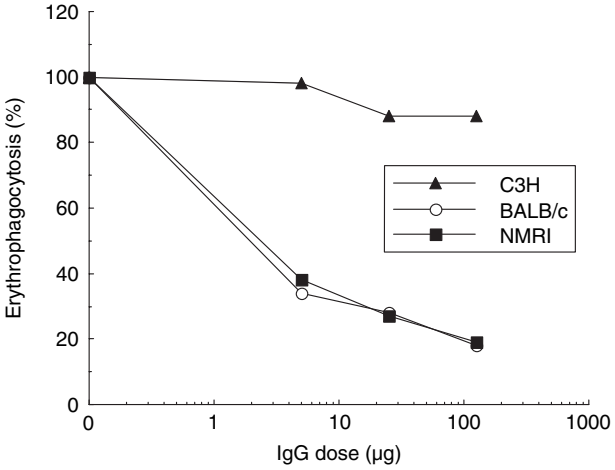

Fig. 4. Inhibition by total IgG of *in vitro* erythrophagocytosis mediated by macrophages from different mouse strains. Pooled peritoneal macrophages from groups of 4–5 BALB/c, NMRI and C3H mice were incubated with erythrocytes sensitized with 34–3C monoclonal antibody (mAb), in the presence of various doses of total IgG, as indicated. Results are shown for each macrophage population as a percentage of erythrophagocytosis observed in the absence of inhibitory IgG.

Variability related to the host

Inhibition of autoantibody-coated erythrophagocytosis by total IgG was tested with macrophages from mice of different genetic backgrounds. With macrophages derived from NMRI and BALB/c mice, a dose-dependent efficient inhibition of autoantibody-coated red blood cell phagocytosis was achieved by total IgG (shown in Fig. 4 for a typical experiment). In contrast, with C3H macrophages, erythrophagocytosis was not suppressed by the same treatment (Fig. 4), although the basal capability of these macrophages to ingest red cells was lower than that of BALB/c and NMRI macrophages (not shown). This mouse strain-related difference was found in five independent experiments.

Finally, the effect of a viral infection of the host on the efficacy of total IgG to inhibit macrophage-mediated erythrophagocytosis was tested by comparing macrophages from control BALB/c mice and from animals infected with LDV. As reported above, the infection enhanced the phagocytosis of 34–3C autoantibody-coated red cells by macrophages (Fig. 1). However, in three independent experiments the suppressing effect of total IgG on erythrophagocytosis that was observed with macrophages from normal, uninfected mice was not found when macrophages from LDV-infected animals were used (Fig. 5). Erythrophagocytosis by macrophages from LDV-infected mice was also less sensitive to the inhibiting effect of IgG3 than phagocytosis by cells from control animals (data not shown).

Discussion

Although many different mechanisms have been proposed to explain the mode of action of IVIg in the treatment of autoimmune diseases [9–21], blockade of Fc receptors on phagocytic cells certainly seems to be involved in its efficiency in reducing autoantibody-mediated anaemia and thrombocytopenia [20,25–31]. It is therefore not surprising that distinct IgG subclasses display widely different abilities to inhibit macrophage-mediated phagocytosis of autoantibody-coated target cells, due to their respective affinity for

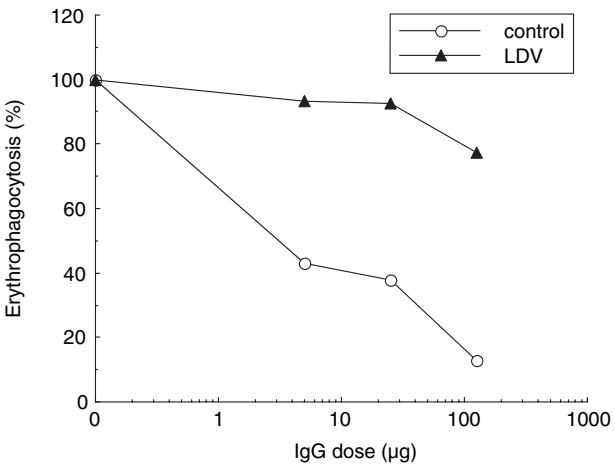

Fig. 5. Inhibition by total IgG of *in vitro* erythrophagocytosis mediated by macrophages from normal and infected mice. Pooled peritoneal macrophages from five control BALB/c mice, and from five BALB/c animals infected with lactate dehydrogenase-elevating virus (LDV) for 3 days were incubated with erythrocytes sensitized with 34–3C monoclonal antibody (mAb) in the presence of various doses of total IgG, as indicated. Results are shown for each macrophage population as a percentage of erythrophagocytosis observed in the absence of inhibitory IgG.

Fcγ receptors expressed on these cells. This may easily account for the strong inhibition of *in vitro* erythrophagocytosis obtained in this study with human IgG1 and IgG3, but not with IgG2 and IgG4, and is consistent with the *in vivo* activity of IgG1 and IgG3 reported previously [36]. This efficiency of IgG1 and IgG3, but not of IgG2 and IgG4, may correspond to binding to FcγRIII that specifically recognizes these isotypes [41] and that is involved in antibody-mediated erythrophagocytosis, at least in the mouse [42].

Because the avidity of IgG oligomers to Fcγ receptors increases with their size [43], it could be postulated that large polymers would be more efficient to block these receptors and therefore to inhibit erythrophagocytosis. Similarly, tetramolecular immune complexes prepared with mouse anti-human IgG monoclonal antibody and human Fc fragments have been shown to inhibit phagocytosis of opsonized erythrocytes efficiently [44]. In the present report, IgG dimers were indeed found to be more effective phagocytosis inhibitors than monomers. However, larger IgG polymers were less efficient than both dimers and monomers. This may be consistent with previous studies, showing that larger IgG polymers are more rapidly internalized and degraded by macrophages with a rapid re-expression of free Fc receptors, while non-aggregating IgG dimers, which are better bound than monomers, are not readily internalized [45,46], and thus might block Fc receptors expressed at the cell surface for longer periods of time. Moreover, IgG3 are more susceptible to form dimers than other IgG subclasses [47] and are actually over-represented in dimers [24] fraction from pooled IgG ([48], data not shown), although it is not established that the increased efficiency of dimers relates only to their isotypic distribution. Thus, the proportion of the IgG3 isotype and/or dimers in IVIg batches may determine their efficacy to inhibit phagocytosis of cellular targets in autoimmune diseases.

Striking differences were observed in the *in vitro* efficacy of IVIg to block phagocytosis when macrophages from various mouse strains were used as effector cells. Macrophages from BALB/c, NMRI and C3H animals could phagocytose autoantibody-sensitized erythrocytes, although at slightly different levels. However, this erythrophagocytosis was strongly prevented *in vitro* by IVIg when BALB/c and NMRI, but not C3H macrophages, were used. This fitted well with preliminary data that indicated a better *in vivo* inhibition of 34–3C-induced anaemia by IVIg in BALB/c and NMRI than in C3H mice (not shown). Thus, genetic factors may determine the efficacy of IVIg to reduce the development of autoantibody-mediated autoimmune diseases. Interestingly, BALB and C3H mice bear distinct Fcγ receptor I alleles [49], but these alleles bind similarly to IgG and should therefore not be responsible for the difference in erythrophogocytosis inhibition by IVIg observed between these two mouse strains. Whether this difference corresponds in the mouse to allotypic polymorphism of human Fc receptor that may affect phagocytosis [50,51] remains to be determined.

Finally, infection with viruses such as LDV or mouse hepatitis virus may increase the severity of autoantibody-mediated autoimmune disease by enhancing the phagocytosis of target cells by macrophages [40,52,53]. Here, we found that the phagocytic activity of these virally stimulated macrophages was hardly inhibited *in vitro* by IVIg. This may be due either to a virally induced increase in Fc receptor expression, that could not be completely blocked by IVIg, or to the involvement of phagocytosis mechanisms independent of Fc receptors that would therefore be insensitive to this type of inhibition. Indeed, although LDV can strongly enhance macrophage-mediated phagocytosis of IgG2a-coated red cells, as well as that of unopsonized erythrocytes, although to a lesser extent (Fig. 1) [40], this effect of viral infection might involve alternative mechanisms such as modulation of complement receptors. Thus, viruses that are frequently involved in autoantibody-mediated autoimmune diseases can decrease the efficacy of IVIg treatment.

In this study, we have shown that factors from both the host and the immunoglobulin preparation may affect the *in vitro* efficacy of IVIg inhibition of target cell phagocytosis by macrophages. In addition to these factors, the nature of autoantibodies involved in diseases such as anaemia may determine the outcome of IVIg treatment, due to the mode of action of these autoantibodies. Indeed, it has been shown that anaemia may be triggered by anti-erythrocyte autoantibodies through diverse mechanisms that may or may not involve phagocytosis [35,36]. In addition, the isotype of these autoantibodies clearly affect their ability to interact with Fc receptors, and thus to induce red cell destruction through phagocytosis [54]. Because, in many cases, the *in vivo* efficacy of IVIg treatment may be correlated with its ability to inhibit *in vitro* target cell phagocytosis ([36], data not shown), it might be of interest to develop such an assay to predict the outcome of this treatment in patients. From our results, it may be postulated that a good predictive value of this type of assay would require the use of both autoantibodies and opsonized target cells and macrophages from the patient himself, in addition to the actual IVIg preparation that would be administered.

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